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Distinctive Developmental Origins and Specificities of Murine CD5$^+$ B Cells

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SUMMARY

CD5$^+$ B cells constitute a small fraction of cells in the spleen of adult mice that exhibit numerous features serving to distinguish them from the bulk of IgD$^+$CD5$^-$ "conventional" B cells. In this review we focus on two major questions relating to this population: 1) the relationship of CD5$^+$ B cells to other B cells; and 2) the distinctive enrichment of particular autoreactive specificities in this subset. The nature of their origins is clarified by a thorough analysis of intermediate stages of early B-cell development in both fetal and adult tissues. The reactivity to bromelain-treated mouse red blood cells serves as a prototype system for the investigation of biased specificities in CD5$^+$ B cells. These lines of investigation lead us to propose that CD5$^+$ B cells in the adult are the remnant of a distinct fetal B-cell differentiation pathway wherein selection of cells from this fetal/neonatal population into the adult long-lived pool results in the over-expression of certain germline-encoded autoreactivities.

INTRODUCTION

B cells in the periphery show considerable heterogeneity when analyzed for expression of IgM and IgD (Hardy et al. 1982). Most spleen B cells and essentially all lymph node B cells in normal healthy animals maintained under specific pathogen-free conditions fall in an IgM$^+$IgD^{++} fraction, but a minor fraction of IgM^{++}IgD$^+$ cells is consistently found in spleen. Based on their similarity to the phenotype of B cells found in neonatal mice, these cells have often been regarded as "immature", recent migrants from the bone marrow. While such cells undoubt-

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cells do fall within this IgM/IgD fraction, other criteria and other surface markers can discriminate more mature cells with this phenotype, such as marginal zone B cells. One cell type with this distinctive IgM/IgD ratio is delineated by expression of CD5, constituting 1-2% of spleen.

B cells that bear the pan-T cell glycoprotein CD5 (originally known as "Ly-1") show a variety of novel features that distinguish them from the bulk of IgD$^+$ CD5$^-$ "conventional" B cells (reviewed in Hayakawa & Hardy 1988, Kipps 1989, Kantor & Herzenberg 1993, Hardy & Hayakawa 1993). Among these are distinctive surface phenotype (IgM^{+++}/IgD^{low}/B220^{low}), novel anatomical localization (enrichment in the peritoneal cavity), early appearance in ontogeny, secretion of certain autoreactive antibodies and increased frequency in several autoimmune mouse strains. Further work has demonstrated predisposition to unregulated growth or even lymphoma. The demonstration of a homologous population in human ("CD5$^+$ B cells") with many similar features, including early appearance and novel biases in specificity together with the long-recognized presence of CD5 on B-lineage chronic lymphocytic leukemia cells (Bournsell et al. 1978) has stimulated interest in defining more precisely the relationship of this B cell subpopulation to the majority of conventional B cells.

In this review we focus on two issues: 1) defining the origins of CD5$^+$ B cells by a thorough analysis of intermediate stages of early B-cell development in both fetal and adult tissues; and 2) studying the expression of particular specificities and associated immunoglobulin V genes enriched in this population. We present the experimental background that supports our current understanding of CD5$^+$ B cells as the remnant of a distinct fetal B-cell differentiation pathway and suggest that the selection of cells from this fetal/neonatal population into the adult long-lived pool results in the over-expression of certain germline-encoded autoreactivities, such as antibody to bromelain-treated mouse red blood cells and intact thymocytes.

B-CELL DEVELOPMENT

Bone marrow is considered the primary site of B lymphopoiesis in adult mice. Surprisingly, several years ago we found that CD5$^+$ B cells were poorly generated from cell transfer of adult bone marrow, in contrast with good repopulation using early B-cell progenitor sources such as fetal or newborn liver (Hayakawa et al. 1985). More recently (Hardy & Hayakawa 1992), we compared SCID mice (that lack lymphocytes of their own; Bosma 1989, Hardy et al. 1989) reconstituted using fractions sorted to enrich for hematopoietic stem cells (Muller et al. 1986, Spangrude et al. 1988) and again found CD5$^+$ B cells when liver was used, but few when bone marrow was the source (Fig. 1). Our initial expectation was that two distinct sets of B cells were being generated in fetal liver, but that gradually one of these differentiation pathways or "lineages" diminished with age and

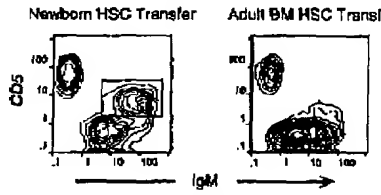

Figure 1. Differential repopulation of CD5$^+$ B cells using a hematopoietic stem cell-enriched fraction sorted from lives of newborn mice versus a comparable fraction sorted from bone marrow of adults. 10^4 Thy-1^{low}/Lineage$^-$ cell fractions (0.2% of total) were isolated and injected i.v. into SCID mice irradiated (300R) the previous day. Two months later animals were sacrificed and cells washed out of the peritoneal cavity were stained and analyzed by flow cytometry with indicated reagents. Other tissues examined (thymus, bone marrow, spleen) were completely reconstituted with conventional B cells and T cells.

eventually became almost completely inactive in adult bone marrow. We sought to investigate this more completely by first clearly delineating the intermediate stages of early B-lineage differentiation in adult bone marrow and then comparing this to the process in fetal liver.

B-lineage differentiation in the bone marrow

Our approach has been to utilize flow cytometry multiparameter analysis using a set of reagents recognizing cell surface molecules differentially expressed during B-lineage development to discriminate intermediate stages in bone marrow (Hardy et al. 1991). Initially, we resolved B220$^+$IgM$^-$ B-lineage cells in mouse bone marrow into four fractions based on differential cell surface expression of determinants recognized by S7 (anti-leukosialin, CD43; Baecher et al. 1988, Gulley et al. 1988), BP-1 (Wu et al. 1990), and 30F1 (anti-heat stable antigen, HSA; Kay et al. 1990). Clear functional distinctions emerged by comparing these fractions: the largest fraction, lacking CD43, consisted of small cells that did not proliferate in Whitlock-Witte type stromal cell culture (Whitlock et al. 1987), whereas the CD43$^+$ fractions, provisionally termed Fr. A, Fr. B and Fr. C, could proliferate and differentiate in stromal culture (Hardy et al. 1991). Phenotypic alteration during such culture suggested an ordering of these stages from Fr. A to Fr. B to Fr. C and thence to CD43$^-$ small pre-B cells (see Fig. 2). Furthermore, functional analysis of sorted fractions demonstrated that the proliferation of these fractions changed from absolute contact dependence in Fr. A to contact independence in Fr. C (Fig. 3), consistent with progression from A to C since altered growth requirements after heavy chain rearrangement have been suggested previously (Sauter & Paige 1988). B220$^+$CD43$^+$ cells in bone marrow can be divided into

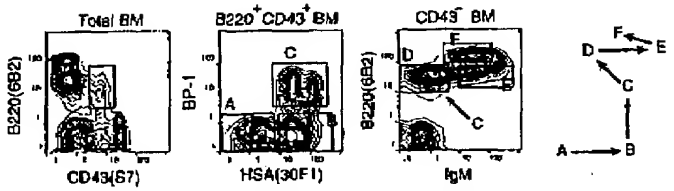

Figure 2. Diagram of B-cell differentiation in BM as resolved by correlated expression of B220(6B2), CD43(S7), BP-1, HSA(30F1) and IgM. Phenotypic transition *in vitro*, differences in functional activity and immunoglobulin gene rearrangement status all support the pathway diagrammed at the right hand side.

TABLE I
Heavy-chain rearrangement in B-lineage cells in bone marrow

	Fractional Retention of Germline			
	fragment 5′ of D		fragment 5′ of J	
Cell fraction	mean	(SE)	mean	(SE)
Fr. A	1.05	(0.06)	0.95	(0.03)
Fr. B	0.98	(0.03)	0.39	(0.02)
Fr. C	1.00	(0.04)	0.17	(0.03)
Pre-B	0.50	(0.01)	0.05	(0.01)
B cell	0.50	(0.04)	0.05	(0.01)

three fractions using an alternate four-color separation with B220, CD43, IgM and IgD. Small pre-B cells (Fr. D) lack IgM and IgD, newly generated B cells (Fr. E) express only IgM and mature B cells (Fr. F) express both IgM and IgD.

Analysis of Abelson-transformed B-lineage cell lines has demonstrated an ordering of Ig recombination, first heavy chain D-J, followed by complete VDJ and finally light chain rearrangement (Yaoita et al. 1983, Alt et al. 1984). Therefore, it was important to characterize the status of immunoglobulin heavy chain gene rearrangement in these fractions since this provided an independent test for our ordering of the stages. We designed a polymerase chain reaction (PCR) amplification assay using pairs of oligonucleotide primers specific for regions 5′ of J_H1 and DFL16.1, sequences that are deleted upon rearrangement of D to J and V to D J (Hardy et al. 1991). By amplifying these sequences together with an α-actin gene segment (which serves to normalize for DNA loading and amplification efficiencies), we could measure levels of D to J and V to D-J rearrangement by quantitating decreases in the intensities of the corresponding fragments (which are resolvable on an agarose gel). Using this procedure we

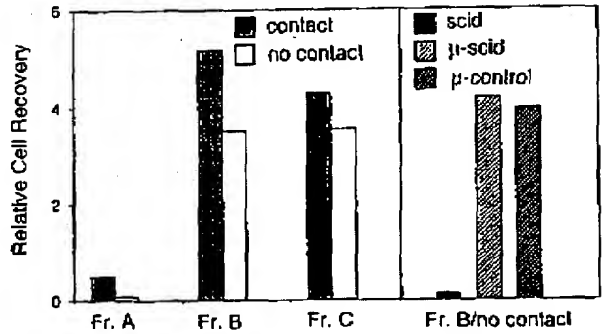

Figure 3. The left panel presents functional differences in B220+CD43+ fractions in bone marrow revealed by culture directly on the FLST2 stromal layer (labelled "contact") or else in a diffusion chamber (labelled "no contact") suspended above the stromal layer. While Fr. A is absolutely contact-dependent for survival, Fr. C is essentially contact-independent. Cells (5×10^4) sorted directly into established cultures and analyzed 4 days later. The right panel presents a comparison of Fr. B cells isolated from SCID and μ-transgenic SCID, supporting the model that productive Ig heavy chain rearrangement is necessary for the transition to contact-independent growth.

found that the Ig genes of Fr. A were in germline configuration, whereas those of Fr. B and C showed increasing D–J rearrangement, but no V–D–J (Table I). In contrast with these Pro B fractions, complete V–D–J rearrangements was seen in CD43− B lineage fractions (pre-B and B) in bone marrow.

Functional changes induced by immunoglobulin heavy chain expression

The alteration in growth dependence on contact-mediated signal(s) occurs at about the point that cells are completing heavy chain rearrangement. We have investigated this issue by comparing the growth of Pro B-cell fractions isolated from bone marrow of immunodeficient SCID mice with Pro B cells isolated from a SCID mouse bearing a rearranged immunoglobulin μ transgene. The defect in SCID prevents the generation of significant numbers of productive Ig rearrangements and so Pro B cells in these mice initiate, but rarely complete, productive heavy chain recombination. We found that Fr. B cells from SCID could survive and even expand in conventional stromal cell culture, but that these cells could not grow (or even survive) in a diffusion chamber culture that allows soluble factors to reach the Pro B cells, but prevents direct contact with the stromal layer (Reichmann-Fried et al. 1993). Interestingly, Pro B cells iso-

luted from μ-transgenic SCID bone marrow could proliferate in diffusion chamber culture, at about the level seen with control μ-transgenic Pro B cells (Fig. 3). Thus it appears that expression of a productive Ig heavy chain signals the early B-lineage cell to alter its growth requirement, and proliferate strongly in response to soluble mediators. We found that growth comparable to diffusion chamber culture was obtained with recombinant IL-7 alone, which confirms the critical importance of this cytokine in B-cell development. A proliferative burst just after the stage of productive heavy chain rearrangement is reasonable in that such an expanded clone of cells would then initiate independent light chain rearrangements, generating a distinct set of immunoglobulin combining sites by combinatorial diversity.

Gene expression in bone marrow B-lineage subsets

A further test of this model of B-cell development has been the analysis of gene expression in these fractions. We utilized the polymerase chain reaction to amplify cDNA synthesized from total RNA isolated from these sorted bone marrow cell fractions in order to determine the level of expression of a set of B lineage-associated genes (Li et al. 1993). The genes analyzed included MB-1, TdT, Lambda-5, Rag-1, BCL-2 and β-actin (which serves as a normalizing control for the amount of RNA and the efficiency of cDNA synthesis). As shown graphically in Fig. 4, this analysis reveals that: 1) MB-1, a molecule associated with immunoglobulin and critical for surface expression and signal transduction (Hombach et al. 1988, Lin & Justement 1992), is present from early stages of the B lineage; 2) terminal deoxynucleotidyl transferase (TdT), an enzyme that mediates addition of nucleotides at the D-J and V-D junctions during heavy chain recombination (Landau et al. 1987), is expressed only during stages where such rearrangement is occurring, Pro B; 3) Lambda-5 and VpreB, that together form a light chain-like molecule that associates with heavy chain prior to light chain rearrangement (Kudo et al. 1990, Kudo et al. 1992), are largely restricted to stages prior to light chain rearrangement; 4) Rag-1 and Rag-2, "recombinase-activating genes" critically important in the process of Ig rearrangement at both heavy and light chain loci (Schatz et al. 1989, Oettinger et al. 1990), are present in both Pro B and Pre-B cell stages; and 5) BCL-2, an oncogene important in maintaining the long-term survival of lymphoid cells (Vaux et al. 1988; McDonnell et al. 1989, Sentman et al. 1991, Strasser et al. 1991a, 1991b), is present at highest levels very early and then again late in the B-lineage pathway, but is found only at low levels in actively differentiating fractions.

One can draw several conclusions from these results. First, the expression of several of these genes confirms the proposed pathway of B-cell development: MB-1 is expressed from the earliest fractions, the surrogate light chain com-

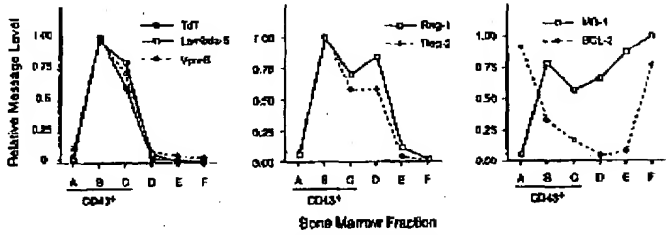

Figure 4. Gene expression in bone marrow as determined by RT-PCR. Message level is reported relative to the maximum value obtained in the analyses. Total RNA was extracted from 10^5 cells sorted directly into guanidinium thiocyanate lysis solution and then cDNA was synthesized using random hexamers and M-MLV reverse transcriptase. cDNA was amplified by PCR using the specific primers as described in (Li et al. 1993). Aliquots were withdrawn at 22 and 26 cycle for separate analysis to ensure that amplification was within the linear range. To verify that equal amount of RNA were added in each PCR reaction, the "housekeeping gene" β-actin was also amplified. Samples were separated by 1.5% agarose gel electrophoresis, blotted and then hybridized with riboprobes prepared from cloned PCR products. Membranes were quantitated using a two-dimensional scintillation detector (Ambis, San Diego, CA).

ponents precede light chain rearrangement and the recombinase-activating genes are expressed in the three stages where heavy and light chain rearrangement are ongoing. Second, the similar pattern of expression seen with TdT, Lambda-5 and VpreB is consistent with the comparable promoter structure shared by these genes (lacking a TATA-box; Lo et al. 1991). Third, the absence of TdT-mediated N-regions from the junctions of light chains is simply accounted for by the precise restriction of TdT expression to stages where heavy chain, but not light chain, is occurring. Finally, the pattern of Bcl-2 is highly reminiscent of that seen with T-cell development in the thymus in that gene expression decreases strikingly at the immature receptor-positive stage, a point where selection and apoptosis is likely to be taking place.

In summary, it is possible to resolve six B-lineage fractions in the bone marrow of adult mice based on expression of CD45R(B220/6B2), CD43(S7), HSA(30F1), BP-1, IgM and IgD. Three CD43(S7)+ fractions represent very early B-lineage cells before or just at the stage of complete heavy chain rearrangement. The status of Ig rearrangement can be analyzed by both deletional and generational approaches, showing that the bulk of cells in Fr. B are D-J, but not V-DJ rearranged (Hardy et al. 1991, Ehlich et al. 1993, Li et al. 1993). Three CD43(S7)− fractions consist of Pre-B, immature B and mature B cells. Again, PCR rearrangement analysis shows that heavy chain rearrangement is complete by Fr. D, and that light chain rearrangement is largely restricted to the CD43− fractions (Hardy

et al. 1991, Ehlich et al. 1993, Li et al. 1993). Application of RT-PCR demonstrates tightly regulated gene expression that supports this model for B-cell differentiation and reveals further aspects of B-lineage associated gene regulation during lymphopoiesis in the bone marrow (Li et al. 1993). These results are presented diagrammatically in Fig. 5.

B-lineage differentiation in fetal liver: a developmental switch

We next sought to compare B lymphopoiesis in bone marrow and fetal liver. We reasoned that transfer of Pro B cell fractions might reveal restricted repopulations that would be obscured when using stem cells. That is, these more differentiated cells, if capable of giving rise to significant numbers of B cells in transfer assays, might already be committed to generating distinctive progeny. Transfer of Pro B cells sorted from bone marrow into sublethally irradiated immunodeficient SCID mice gave rise to B cells, but not T cells (Hardy & Hayakawa 1992). Furthermore, the limited numbers of cells used in these experiments (5×10^4–10^5 per mouse) did not establish long-lasting B lymphopoiesis. Rather, after limited expansion, the cells differentiated into non-proliferating mature B cells which could be found in the spleen and peritoneal cavity.

Flow cytometry analysis of fetal liver revealed that essentially all B-lineage cells (defined as B220$^+$) were CD43$^+$ at day 15/16 of gestation. That is, it appears that most B-lineage cells at this time are at the Pro B or earlier stage. Using the PCR rearrangement assay described above, such cells can be shown to possess extensive D–J, but not V–D–J, rearrangement consistent with their designation as "Pre B".

We then compared SCID recipients repopulated with such committed progenitors from bone marrow or fetal liver and found that distinctively different B cells were generated from Pro B cells present at these two developmental times. As early as 7–9 days following i.v. injection of these progenitors, B lymphocytes can be recognized in peripheral blood as B220$^+$IgM$^+$ cells. All recipients of fetal progenitors show CD5 expression on at least some of these PBL B cells, whereas CD5 expression is not found on the B cells repopulated in recipients of bone marrow progenitors. By 2 to 3 weeks after transfer, B cells are readily detectable in the spleen and peritoneal cavity (PerC). These spleen or PerC B cells repopulated from bone marrow progenitors appear "conventional" in phenotype (IgD^{++} and CD5$^-$) and show peak repopulation 2–3 weeks after transfer, decreasing thereafter. In contrast, B cells repopulated from fetal liver progenitors have an IgM^{++}/IgD^{low} phenotype and many express CD5. This population increases to maximal levels by 2 months after transfer, thereafter remaining constant. Representative flow cytometry analyses are shown in Fig. 6. These results prompt us to suggest that essentially all of the B-lineage cells early in development are committed to a distinct developmental pathway, eventually giving rise to a

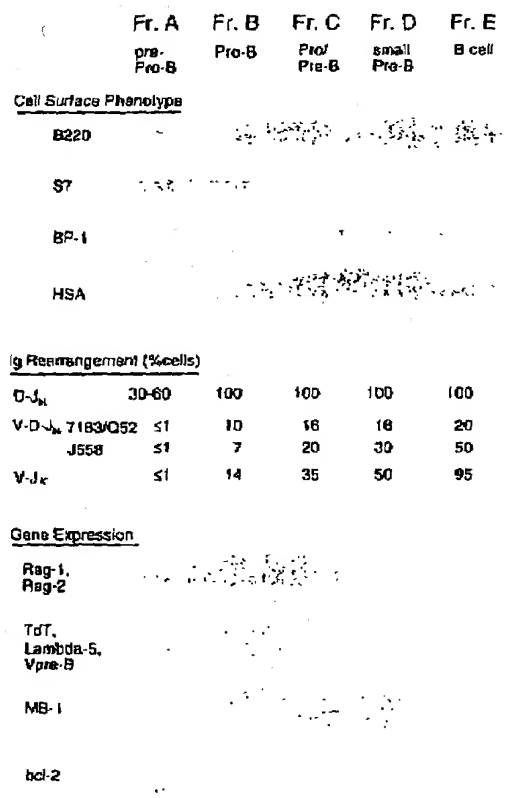

Figure 5. Diagram of bone marrow B-cell development showing cell surface phenotype, rearrangement status and gene expression in intermediate stages discussed in this review. The higher early rearrangement reported here was determined by positive PCR analysis (Li et al. 1993).

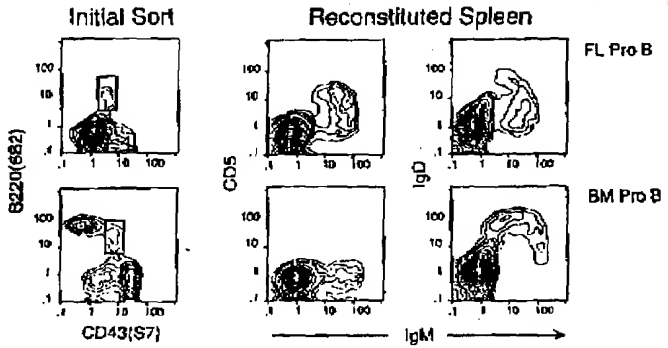

Figure 6. Distinct B-cell phenotypes reconstituted 3 weeks after transfer of Pro B cell fraction from fetal liver and adult bone marrow. Pro B cell fractions (1–2% of total cells) were isolated from day-16 fetal liver and bone marrow of adult (>3 month) mice by cell sorting, then injected i.v. into SCID mice (1×10^5/animal) irradiated 300R the previous day. Mice were sacrificed 3 weeks later and cells from spleen were stained and analyzed as indicated. Bone marrow and thymus showed essentially no engraftment.

phenotypically resolvable population of B cells, many of which are CD5$^+$ (Hardy & Hayakawa 1991).

This result contrasts with our earlier expectation that two distinct pathways would be present in fetal liver and that one would diminish with age. Instead we see evidence of a "developmental switch" in B lymphopoiesis, from a fetal type early on to an adult type in bone marrow by 2–3 months of age.

Gene expression in fetal liver B-lineage fractions

If the destiny of B-lineage cells in identical microenvironments differs depending on the Pro B cell source, then this implies that gene expression at the Pro B cell stage should already be different. Therefore, we have begun to use the RT-PCR assay described above for bone marrow B-lineage fractions to analyze fetal liver in order to understand the mechanism for generation of the phenotypically distinct mature B-cell populations that we observed in the SCID transfer experiments. Our initial results (Fig. 7) are quite striking: whereas many B-lineage genes are expressed in fetal liver at levels comparable to the Pro B cell stage in bone marrow, two genes analyzed (TdT and the precursor lymphocyte regulated myosin-like light chain, PLRLC) are not. Both of these genes are highly expressed in Fr. B of bone marrow but absent from fetal liver. TdT mediates N-nucleotide addition

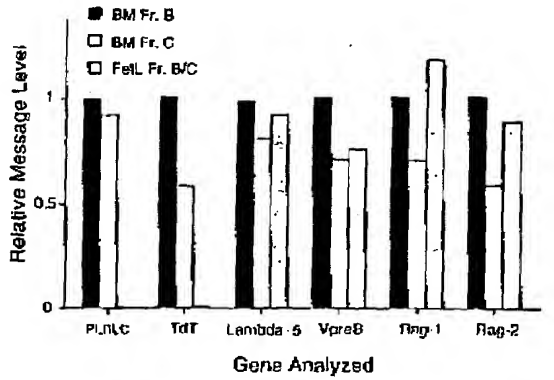

Figure 7. Gene expression in fetal liver compared to bone marrow as determined by RT-PCR. Both TdT and PLRLC are highly expressed in Pro B fractions from bone marrow (BM), but are absent from Pro B cells of fetal liver (FetL). In contrast, Lambda-5, VpreB, Rag-1 and Rag-2 are expressed at comparable levels in both fractions. For fetal liver, Fr. B and C were not resolved. Analysis was performed as described in Fig. 4.

at the D–J and V–D junctions and decreased expression in fetal tissues had been reported previously using immunofluorescence techniques. However, we show essentially a complete absence from purified Pro B cells that are otherwise quite similar to the homologous fraction in adult bone marrow. PLRLC was recently described as differentially expressed in precursor lymphocytes from adult and fetal sources (Oltz et al. 1992) and our analysis confirms that this is also the case for purified Pro B cells.

In contrast to this differential expression of TdT and PLRLC, other genes critical to B-cell development such as Rag-1/2 and VpreB/Lambda-5 are comparably expressed. Thus, it appears that the homologous fractions from fetal liver and bone marrow share a number of features (ongoing D–J rearrangement, ability to grow on a support stromal layer and expression of genes such as Rag-1 and Lambda-5), but also exhibit differences in expression of other genes and give rise to phenotypically distinct sets of mature B cells in identical SCID recipients. Absence or decreased levels of TdT will reduce CDR3 diversity and conceivably could result in increased homology-mediated recombination (favoring certain V–D or D–J combinations). This could lead to a difference in the repertoire of antigen specificities expressed by the fetally-generated B-cell population compared to adult B cells. The function of PLRLC is unknown at present, but homology to a structural gene (myosin light chain) suggests a role in the cytoskeleton so

that differential expression might mean that the coupling of surface molecules to intracellular proteins might differ. It is reasonable to predict that expression of TdT and PLRLC will not be the only differences between fetal and adult B lymphopoiesis responsible for the distinct mature B-cell phenotypes.

Expression of CD5 in B-cell development

Expression of CD5 as determined by surface immunofluorescence on the cell sorter serves to mark a functionally distinct subset of B cells so it is interesting to determine when CD5 is first detectable during B-cell development. By surface immunofluorescence, CD5 is undetectable on B-lineage surface Ig$^-$ cells in both fetal liver and bone marrow. Furthermore, culture of fetal liver Pro B generates IgM$^+$ cells, and a fraction of these begin to express CD5, whereas the IgM$^+$ cells in these cultures are CD5$^-$. Thus, CD5 expression would appear to be a relatively late-appearing marker during B-cell differentiation, in contrast to its expression early in T-cell development.

We have also analyzed CD5 message expression using an RT-PCR assay. The results, shown in Figure 8, reveal one difference from the surface immunofluorescence analysis: CD5 message is detectable at the earliest stages of B-cell differentiation (Pro B and Pre-Pro B) in both bone marrow and fetal liver, but decreases strikingly during bone marrow B-cell differentiation. Similar analysis of peripheral B-cell subsets shows good correspondence between message levels and surface expression assessed by flow cytometry. We do not as yet understand the significance of the discordant CD5 expression early in B-cell development, but speculate that it could be cytoplasmic-restricted at low levels that would be difficult to demonstrate by standard protein analysis techniques.

BIASED SPECIFICITIES AND Ig V_H GENE USAGE IN CD5$^+$ B CELLS

One of the most distinctive features of the CD5$^+$ B-cell subset is its association with certain types of autoantibody. Thus, CD5$^+$ B cells are uniquely elevated in the autoimmune NZB mouse strain (Hayakawa et al. 1983) and most of the cells in severely autoimmune motheaten strain are CD5$^+$ (Sidman et al. 1986). In addition, "natural" autoantibodies such as antibody to bromelain-treated mouse red blood cells (BrMRBC) and anti-thymocyte autoantibody (ATA) were shown early on to be distinctively enriched in CD5$^+$ B cells, even in normal mouse strains (Hayakawa et al. 1984). In this review we describe our work on antibody to thymocytes and to BrMRBC.

The anti-bromelain-treated red blood cell specificity

A difference in the antibody repertoire between these two B-cell subsets (CD5$^+$ IgD^{low} and CD5$^-$IgD^{++}) in adult mice was first suggested by exclusive anti-

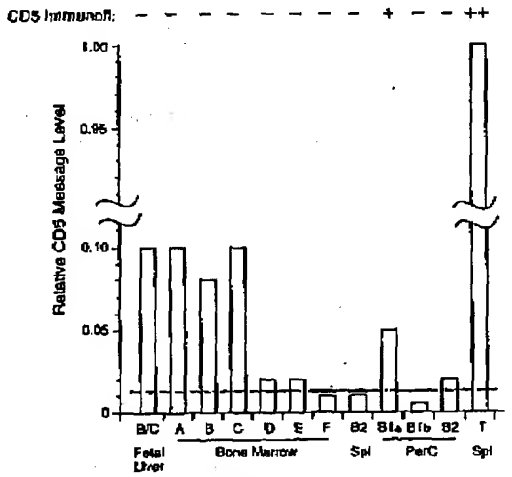

Figure 8. CD5 RT-PCR expression analysis showing discordant CD5 message and immunofluorescence cell surface expression. RT-PCR analysis was performed as described in Fig. 4. For peripheral cells, B1a refers to IgM^{++}IgD^{+}CD5$^+$ cells, B1b means IgM^{++}IgD^{+}CD5$^-$ cells and B2 is IgM^{+}IgD^{++}CD5$^-$. All values are given relative to CD5 expression in spleen T cells (=1.00). Immunofluorescence analysis of CD5 presents relative surface expression detected by the 53-7 rat anti-CD5 antibody. The grey line indicates background level of detection by RT-PCR.

BrMRBC (bromelain-treated mouse red blood cells) autoantibody secretion from CD5$^+$ B cells (Hayakawa et al. 1984). This was demonstrated by using both BALB/c and SM/J mice. Results showed that depletion of CD5$^+$ B cells from the spleen specifically eliminated production of this autoantibody and conversely, that purified CD5$^+$ B cells uniquely exhibited anti-BrMRBC secretion elicited by LPS (Hayakawa et al. 1984, Hardy et al. 1989). The screening of hybridomas generated from sorted CD5$^+$ B cells and CD5$^-$IgD^{++} B cells confirmed that the CD5$^+$ B cell population was indeed enriched for cells with this specificity (Fig. 9). As mentioned above, by comparing hybridomas generated from splenic CD5$^+$ B cells and CD5$^-$ B cells sorted from SM/J strain mice we found that anti-BrMRBC-producing hybridoma clones comprised 1–2% of the hybridomas from CD5$^+$ B cells, but only 0.03% in hybridomas made from CD5$^-$IgD^{++} B cells (Hardy et al. 1989).

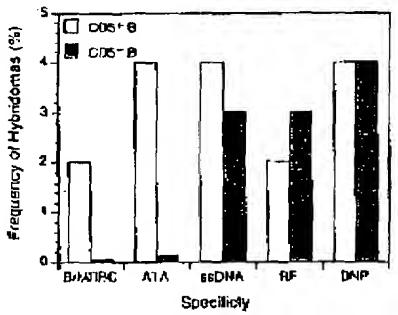

Figure 9. Anti-Br MRBC and ATA reactivity is enriched in CD5 B-cell derived hybridomas, whereas other classes of autoreactivity such as DNA or rheumatoid factor (RF) are not.

V_H gene usage in anti-BrMRBC hybridomas; V_H11 and V_H12

A gene from the V_H11 family is predominantly utilized in anti-BrMRBC B cells in NZB and C3H mice (Reininger et al. 1987). We found that this was also the case for SM/J mice. Sequencing the heavy chain V-genes of two anti-BrMRBC hybridomas (Hardy et al. 1989) from SM/J showed repeated usage of this gene in association with a member of the V_k9 family. We cloned the V_H11 gene and found, using Southern analysis, that 14/16 of our anti-BrMRBC hybridomas possessed V_H11 rearrangements. Using the cloned gene we showed that the highly homologous V_H genes were very few in number (2–3) so that V_H11 defines one of the smallest V_H families. Also, analysis of DNA from mouse lines that had undergone recombination within the V_H locus showed that both of these V_H11 genes mapped upstream of the S107 cluster. This means that V_H11 is not among the genes located proximal to D, genes that have been suggested to be over-represented early in development. The small fraction of anti-BrMRBC hybridomas that did not utilize V_H11 (2/16) had rearranged another novel V_H gene, V_H12 (Pennell et al. 1989). This family, with only one member, was originally described in a CD5$^+$ B lymphoma (Pennell et al. 1988) and has also been seen in hybridomas generated from B cells washed out of the peritoneal cavity, a rich source of CD5$^+$ B cells (Mercolino et al. 1989).

V_H11 rearrangement in normal B cells

This enrichment of V_H11-encoded anti-BrMRBC specificity in the CD5$^+$ B-cell subset was also demonstrated with normal cells of BALB/c mice by analysis of

DNA rearrangement. Using PCR, we found enriched V_H11J_H1 rearrangement in Ly-1 B cells from both spleen and peritoneal cavity as compared to either bone marrow pre-B cells or adult B cells (IgD^{++}/CD5$^-$) from either spleen or PerC (Carmack et al. 1990). We designed oligonucleotide primers homologous to the V_H11 leader sequence and J_H1 (the rearrangement seen in all hybridomas analyzed) and carried out amplification together with a set of primers that amplified another region of DNA upstream of Cμ that served to normalize the V_H11 signal (see Fig. 10). Our data (Table II) clearly demonstrated a difference in repertoire between CD5$^+$ and CD5$^-$ B-cell subsets, even when located in the same tissue. They also showed that V_H11 does rearrange in bone marrow (and its progeny, the IgD^{++} cells of spleen) at about the frequency that would be expected for a small V_H family. Thus one explanation for the over-representation of V_H11/anti-BrMRBC in CD5$^+$ B cells, that somehow the promoter/enhancer structure restricts its rearrangement to fetal B cells, appears unlikely. Together with the finding that V_H11 (and V_H12) are not located proximal to D, these results suggest that the initial V gene usage of CD5$^+$ B cells may not be strikingly different from that of bone marrow-derived B cells. Rather, it appears likely that it is selection

Figure 10. Diagram for V_H11 PCR used to generate the data shown in Table II. A V_H11 leader-J_H1 primer combination amplified V_H11 rearranged DNA and simultaneously a separate pair of primers amplified a DNA fragment present in all IgM$^+$ B cells. This normalizing fragment was chosen because it was also present on the cloned V_H11 sequence.

TABLE II
V_H11 rearrangement in B-cell subsets

Cell type	Frequency of cells (%)	V_H11-J_H1 (%)	SE
BM Pre-B	8	0.7	0.1
BM B	10	0.8	0.3
Spl IgD^{++} B	50	0.9	0.1
Spl CD5$^+$ B	2	9.0	1.3
PerC IgD^{++} B	5	1.0	0.1
PerC CD5$^+$ B	26	10.0	1.8
Spl T cells	30	0.2	0.1

operating on the CD5$^+$ B-cell population that results in the extreme biases that are found with certain specificities, particularly autoreactive ones.

Positive selection in CD5$^+$ B cells

The initial repertoire of CD5$^+$ B cells generated during the fetal stage has not been examined as yet. However, a change in this repertoire with ontogeny has been suggested by an increase in the frequency of anti-BrMRBC-specific B cells within the CD5+ B-cell subset. Mercolino et al. found that anti-BrMRBC-specific B cells, detected as PtC (phosphatidyl choline)-binding cells, increased with age during normal ontogeny (Mercolino et al. 1988). We observed that this was also the case during the B-cell reconstitution period for recipients of fetal B-cell progenitors (Hardy & Hayakawa 1991). In contrast, mice reconstituted with bone marrow did not develop a population of BrMRBC-specific B cells, even 1 year after stem cell transfer.

The enrichment of anti-BrMRBC B cells in CD5$^+$ B cells is more evident when analyzing cells in the peritoneal cavity where CD5$^+$ B cells preferentially reside (Hayakawa et al. 1986). More than 30% of IgM$^+$ B cells present in the PerC are CD5$^+$ B cells. Ten percent of CD5$^+$ B cells in the PerC of adult BALB/c mice are anti-BrMRBC B cells, detectable as antigen (PtC)-binding B cells (Mercolino et al. 1988). We found that such B cells are also specifically detected as B cells labeled by anti-BrMRBC antibody itself (Carmack et al. 1990). Cells capable of anti-BrMRBC secretion and bearing V_H11 gene rearrangement are uniquely labeled by anti-BrMRBC antibodies, but not by antibodies with other specificities. This strongly suggests a constant occupancy of the anti-BrMRBC Ig receptor by multivalent antigen which would allow specific "sandwich" staining. Thus one likely reason for preferential selection and maintenance of CD5$^+$ B cells is the availability of antigen. Certain autoantigens may serve as such antigens if corresponding autoantibody specificity affinity can be encoded by germline genes.

Nearly identical V_H11 gene sequences have been found with anti-BrMRBC B hybridomas established from a pool of mice and from several different strains. This suggests that the specificity is germline gene-encoded with no somatic mutation. Further, the increase of anti-BrMRBC specificity in the CD5$^+$ B-cell subset is not simply due to clonal proliferation of one or a few rearrangements. This was suggested by the presence of anti-BrMRBC hybridomas using different J_k (Hardy et al. 1989).

Sequences of V_H11-J_H1 rearrangements from a single mouse

The presence of numerous unrelated clones in the anti-BrMRBC B cell fraction was confirmed by cloning and sequencing of V_H11 to J_H1 rearranged DNA by

PCR amplification from CD5$^+$ B cells of a single BALB/c mouse which revealed many distinct CDR3 regions. These sequences (Fig. 11) showed a relatively diverse set of rearrangements within the constraints already noted in sequence analysis of anti-BrMRBC hybridomas: relatively short CDR3 regions and little N-addition. Half of the clones sequenced have no obvious N-addition, while many of the rest have only short N-sequences at the D-J junctions. This is consistent with the low levels of TdT expected to be present during rearrangement in fetal Pro B cells. However, about a quarter of the sequences show significant N-addition (4 or more bases), suggesting that the precursors of at least some $V_H11^+CD5^+$ B cells derive from Pro B cells expressing higher levels of TdT. Perhaps some cells differentiating along the proposed "fetal pathway" begin to express TdT toward the end of this process (at 1-2 weeks after birth) or else strong selection for BrMRBC-binding cells enriches even rare cells containing some N-addition. One clear point is that 25% of V_H11 rearrangements in CD5$^+$ B cells contain readily detectable N-addition and so the presence such sequences does not block a cell from entering this population. This analysis also shows common clones present in CD5$^+$ B cells in both spleen and peritoneal cavity suggesting a common precursor or else trafficking between the two compartments.

Translation of the nucleotide sequences (Fig. 12) shows evidence of a preference for tyrosines and serines in the V_H11-J_H1 CDR3 region. This may arise partly

V_H11	D		J_H1	No. of Clones		D family
TATATGNCATA			TACTGTTAC	Spl	PerC	
TCTATGNDA	TATGATGGTTAC		TACTGGTAC	5	11	SP2
TCTATGNDA	TACGTTTNTAGC		TACTGGTAC	6	3	FL16
TCTATGAGA	GGTA	GtGg	TACTGGTTC	3	6	SP2
TCTATGAGA	TACGGAGAT		TACTGGTAC	1	2	?
TCTATGNDA	TATGGTTA	tgg	CTGGTAC	2	1	SP2
TCTATGNDA	TACGCAC	gatggc	TACTGGTAC	3	2	SP2
TCTATGNDA	TACGGTTG	c	TACTGGTAC	2	1	FL16
TCTATGAGA	TATGG	ggat	TACTGGTAC	1	2	SP2
TCTATGNDA	TACGGTAGT	tg	CTGGTAC	1	-	FL16
TCTATGNDA	TATGG	ggac	TACTGGTAC	1	-	SP2
TCTATGNDA	TATCGAGGGTAC		TACTGGTAC	1	-	?
TCTATGAGA	TATGACGTTAAC	a	GGTAC	1	-	SP2
TCTATGNDA	TATGGTT	cL	TACTGGTAC	1	-	SP2
TCTATGAGA	TACTACGGTAGTAGCT	tc	TACTGGTAC	1	-	FL16
TCTATGNDA	TATCGGGGTTAC	c	ACTGGTAC	-	4	SP2
TCTATGNDA	TATGGNGGA		TACTGGTAC	-	1	SP2
TCTATGNDA	TATGATGGTTAC		TACTGGTAC	-	2	SP2
TCTATGAGA	TATGATTATGTAGC		TACTGGTAC	-	1	?

Figure 11. Junctional diversity of V_H11-J_H1 rearrangements in CD5$^+$ B cells from a single BALB/c mouse. Nucleotide sequences obtained after PCR amplification of V_H11-J_H1 rearrangements from sorted CD5$^+$ B cells of spleen and peritoneal cavity. DNA prepared from sorted samples was amplified using primers that contained restriction sites to facilitate cloning. Amplified DNA was then cut with appropriate restriction enzymes, cloned and the clones sequenced. Non-germline nucleotides are indicated between the junctions in lower case. The number of instances of each sequence in the spleen and PerC samples is also indicated.

V	D	J
C H R	Y G S	Y W Y
C N R	Y G S	Y N Y
C H R	Y G S	C N Y
C H R	Y G G	Y W Y
C H R	Y G G	Y W Y
C N R	Y D D	Y W Y
C H R	Y G D	F H Y
C H R	Y G N	G N Y
C H R	Y G V	H R Y
C I R	Y G G N	R W Y
C N R	Y D G H	Y N Y
C H R	Y D G Y	Y H Y
C H R	Y D E G	Y W Y
C H R	Y G S S	Y N Y
C N R	Y S S S	Y N Y
C H R	Y R P Y	Y H Y
C H R	L G Y S	Y W Y
C H R	Y Y G E S F	Y N Y

Figure 12. Amino acid diversity in CDR3 of V_H11/J_H1 rearrangements from CD5$^+$ B cells of a single BALB/c mouse. Amino acid translation of the nucleotide sequences shown in Fig. 11. The repetitive occurrences of tyrosine (Y) and serine (S) or glutamate (D) residues probably reflect selection for antigen binding.

from preferential homology-mediated recombination (see below), but probably also reflects antigenic selection. Antigen-mediated selection is also suggested by light chain analysis revealing strong association of a particular V_k9 gene with the V_H11-J_H1 heavy chain in CD5$^+$ B cells. As was mentioned above, the D is short, encoding only 3 or 4 amino acids in most cases.

Genomic sequence of V_H11 gene

Sequence analysis of V_H11–J_H1 rearrangements in hybridomas and normal cells has showed a striking preference for D sequences starting with "TA". Studies of V gene sequences in neonatal B cells where N-regions are infrequent have suggested that homology between the recombination joints can select for particular V–D or D–J rearrangements (Feeney 1990, Gu et al. 1990). In order to resolve this question, we have carried out sequence analysis of one of the copies of the V_H11 genes. This V_H11 sequence was cloned as an EcoRI fragment and an oligo complementary to the first 7 amino acids of the leader (5'-TAAGCTCAGTTC-CCACTCCAT-3) was used to determine the sequence. Fig. 13 shows the 5' flanking region, leader, intron, CDR 1 and 2 and the framework 1, 2, and 3 regions, along with the heptamer/nonamer recognition sequence in the 3' region. Transcriptional motifs, the TATA box, octamer and heptamer are indicated in the 5' flank (see Staudt & Lenardo 1991 for review). An inverted nucleotide region to the TATA box is also shown just upstream of TATA with arrows indicating polarity. The recognition sequences for the recombinase complex that

mediates Ig gene rearrangement are shown at the 3' end along with the 23 base spacer (Ns) in between the heptamer/nonamer. As is clear from the figure, the V_H11 germline sequence indeed encodes two nucleotides in amino acid 95 which normally are assigned to the D gene, TA. This position can only become a tyrosine (TAT or TAC) because the other two nucleotides would result in stop codons (TAA, ochre or TAG, amber). The consensus recognition sequence is shown just below this region with two stars indicating nucleotide differences. Thus it would appear that the D usage in these cells is biased to favor particular recombination points based on this type of homology, generating a tyrosine residue.

The anti-thymocyte autoreactive (ATA) specificity

Positive selection for autoreactive specificities in CD5 B cells is not limited to the BrMRBC/PtC reactivity. Another good example is provided by natural anti-thymocyte autoantibodies (ATA). ATA secretion occurs spontaneously in normal animals (Shimi & Mellors 1971) and while high levels of ATA have been found in animals with autoimmune disease (e.g., NZB mice), they have also been detected at high levels in some strains of mice lacking any obvious pathology (SM/J mice (Eisenberg et al. 1979)). Both NZB and SM/J mice have elevated levels of CD5$^+$ B cells (Hayakawa et al. 1983, Rabinovitch et al. 1986) along with increased levels of anti-thymocyte autoantibodies in their sera which suggested an association. Furthermore, CD5$^+$ B cells in NZB mice are largely responsible for the spontaneous secretion of IgM *in vitro*, including ATA (Hayakawa et al. 1984). However, it was unclear whether this was due to the unique predisposition of NZB CD5$^+$ B cells to secrete immunoglobulin spontaneously *in vitro* or instead to a higher frequency of ATA-specific B cells in the CD5$^+$ B cell population.

Purified CD5$^+$ B cells and CD5$^-$ B cells from SM/J spleen, or BALB/c peritoneal cavity CD5$^+$ B cells and CD5$^-$ B cells from BALB/c spleen were used for comparing levels of ATA secretion in LPS-culture (Hayakawa et al. 1990). As Fig. 14 shows, both supernatants from cultures of CD5$^+$ B cells contain ATA activity. Although CD5$^+$ B cell cultures initially secrete more IgM than CD5$^-$ B-cell cultures, no anti-thymocyte activity was ever detected in the supernatant from CD5$^-$ (conventional) B cells during 7 days of LPS-culture, even after comparable levels of IgM were attained. The possibility that ATA secretion from CD5$^+$ B cells in the LPS culture system might be dependent on T cells (or some other cell type) could be excluded since CD5$^+$ B-cell depleted SM/J spleen cells, which include all non-B cells in spleen, did not show ATA activity during the course of culture (similar to results using purified B-cell populations). Thus, these data strongly suggested that CD5$^+$ B cells are responsible for natural ATA secretion in most strains and, further, that the increased population of CD5$^+$ B

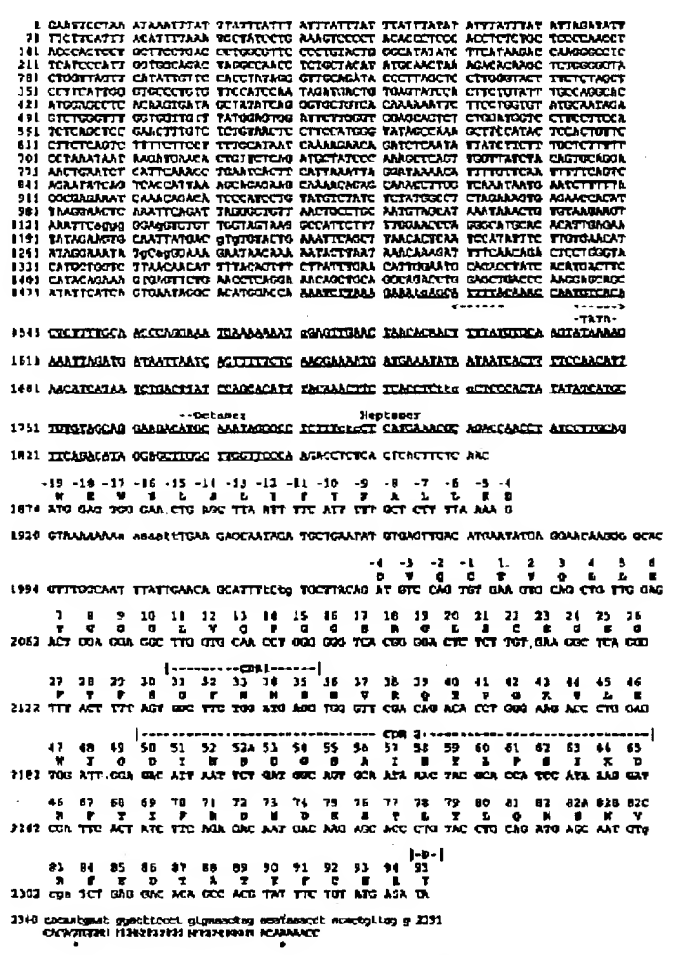

Figure 13. Nucleotide sequence of 2.4 kb of the V_H11 germline gene. Sequence starts from the EcoR1 site (GAATTC). Amino acid translation of coding region is shown above the nucleotide sequence in bold and these are numbered according to Kabat. Underlined region indicates sequence in hybridoma cell line 2-2G4 (Hardy et al. 1989). High molecular weight DNA was isolated from the liver of SM/J mice by conventional methods and digested to completion with *Eco RI*. Digested DNA was electrophoresed in a 1% low melting agarose gel and the V_H11 germline bands around 8.4 kb were cut out and the DNA isolated and cloned into λ-Zap II (Stratagene, La Jolla, CA) and the partial library screened with a V_H11 probe (Hardy et al. 1989). Positives were identified and recovered. λ Zap II was then induced with helper phage to automatically excise the pBluescript Phagemid vector contained within, yielding a plasmid vector. Positive plasmids were recovered and purified plasmid was digested with ExoIII/mung bean nuclease (Stratagene) to produce a series of unidirectional nested deletions of the germline V_H11 gene. Nested deletions were then sequenced using the universal primer contained in the plasmid. Contig fragment sequences were assembled in GCG. Sequence data of two or more overlapping clones are shown as upper case letters. Lower case letters indicate only one clone with readable sequence at that position.

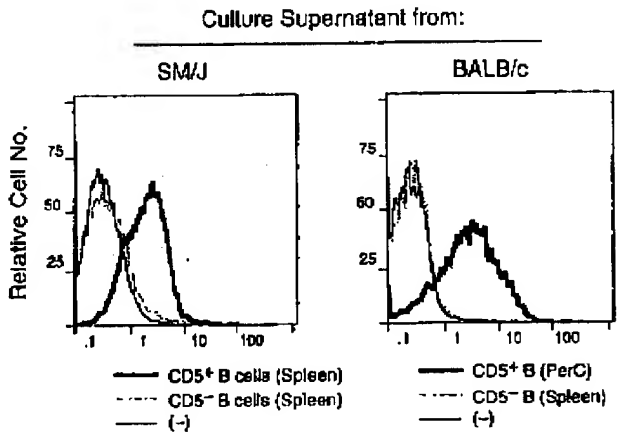

Figure 14. Anti-thymocyte autoantibody activity (ATA) in supernatants of LPS-stimulated sorted spleen or peritoneal washout cell fractions from *SM/J* and BALB/c mice. $CD5^+$ B cells or $CD5^-$ B cells were isolated by cell sorting and cultured. Only $CD5^+$ B cell fractions produced ATA. "–" indicates the autofluorescence background.

cells in SM/J mice is responsible for the characteristically high levels of serum ATA found in this mouse strain.

ATA specificities: carbohydrate determinants dependent on Thy-1 expression

ATA from $CD5^+$ B cells was characterized further by generating hybridomas. Hybridomas made from $CD5^+$ B cells and conventional $CD5^-(IgD^{+/+})$ B cells

sorted from a pool of SM/J mouse spleen were plated in limiting dilution to assess the frequency of clones/well as described previously (Hayakawa et al. 1988). As described above (Fig. 9), the initial screening of 576 IgM-secreting wells from both CD5$^+$ B cells and CD5$^-$ B cell fusions showed that the frequency of clones with anti-thymocyte binding reactivity was at least 10 times higher in CD5$^+$ B cells, consistent with results of the ATA-secretion assay described above. Further analysis of such hybridoma clones revealed several distinct ATA binding profiles to different cell types (Hayakawa et al. 1990). Most monoclonal IgM ATA from CD5$^+$ B cells selected for thymocyte binding activity showed specific binding to T-lineage cells (among hematopoietic cells tested). However, their specificities appeared diverse, exhibiting differential reactivity to thymocytes at various maturational stages, variable binding to peripheral T cells (CD4$^+$ and/or CD8$^+$), and different degrees of species crossreactivity (mouse/rat). Most reacted with thymocytes from all strains of laboratory mice tested including SM/J, the strain from which the hybridomas were derived.

The reactivity of 11 of these autoantibodies was sensitive to prior treatment of target cells with sodium metaperiodate which preferentially oxidizes terminal sialic acid groups, implicating carbohydrate as an important constituent of the antigenic determinant (Hayakawa et al. 1990). Further, as Thy-1 is a major glycoprotein of the thymus, we investigated the ability of anti-Thy-1 antibody to block ATA binding. The binding of eight ATA antibodies was nearly completely blocked by prior or simultaneous incubation of thymocytes with anti-Thy-1 antibody, while the binding of two other ATA antibodies was partially blocked (Fig. 15). Blocking by anti-Thy-1.1 or anti-Thy-1.2 antibodies was specific to the appropriate Thy-1 allele and other antibodies (anti-CD45, anti-CD5) which react with determinants present on the majority of thymocytes did not block. This result suggests that a determinant detected by many of these antibodies either resides on the Thy-1 glycoprotein or on a molecule that is present in close proximity to Thy-1. Indeed, reactivity was lost by a cell line selected for absence of cell surface Thy-1 expression and restored upon transfection of this line with the Thy-1 gene. Several other ATA, such as 2-4C10, were found to react with glycolipids unrelated to Thy-1.

V_H gene usage in ATA hybridomas

The differential staining profiles, reactivities and Thy-1 inhibition of these ATA hybridomas suggested that their antigenic determinants could be quite diverse. This was confirmed by sequencing the immunoglobulin V genes of these hybridomas (Hayakawa et al. 1990). We found that 14 Ly-1 B-derived ATA hybridomas utilized V_H-genes belonging to four distinct families (J558, 3609, Q52, Vgam3.8). Notably, our data revealed that fine specificities definable by our assays largely correlated with usage of particular V_H genes. Three groups of ATA used nearly

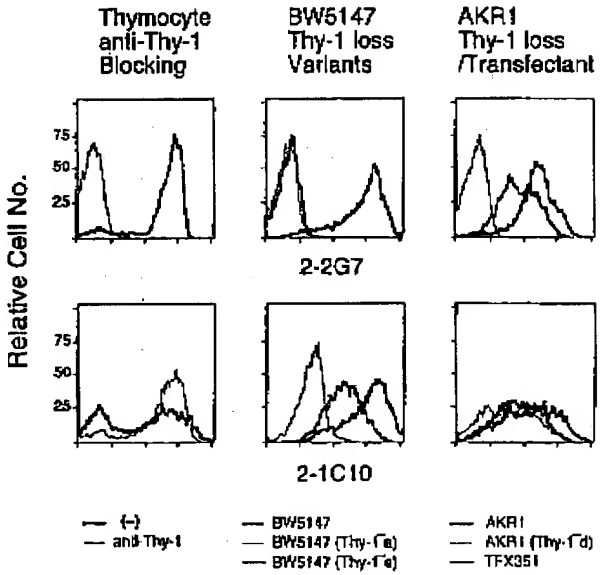

Figure 15. Examples of ATA hybridomas recognizing two distinct epitopes. One (2-2G7) is completely blocked by anti-Thy-1 antibody, whereas the other (2-1C10) is unaffected. 2-2G7 binding is also lost on BW5147 Thy-1 variant lines whereas 2-1C10 staining is not. Finally, transfection of a Thy-1 gene into an AKR Thy-1 variant line (TFX35I) restores 2-2G7 staining. All these results suggest that 2-2G7 is reacting with a determinant on (or very closely associated with) the Thy-1 glycoprotein.

identical V_H genes, one utilizing 3609, another with a J558 member and a third with a different J558 gene. Two hybridomas derived from a clonal expansion since they had identical V–D–J sequences. However, we also found repetitive usage of an identical V_H gene not due to clonal proliferation. The four 3609 hybridomas are unrelated, as shown by D and J_H differences. Another pair used identical V_H and J_H genes, but were unrelated, as distinguished by their D genes. In conclusion, repetitive V_H gene usage in ATA hybridomas is due both to clonal expansion and to independent incidence and is consistent with antigenic selection. It is worth noting that repetitive usage of particular Ig V genes by CD5$^+$ B cells has been reported previously by others (Forster et al. 1988, Stall et al. 1988, Tarlinton et al. 1988).

CONCLUDING REMARKS

Our current understanding of CD5$^+$ B cells is that they are the progeny of a "distinct lineage" of B-cell differentiation on the basis of developmental origin. As shown by differences in the levels of CD5 and IgD, we presume that the control of expression of several genes will differ significantly between these B-cell lineages. Our data also suggest that Ly-1 B cells, generated early in ontogeny, are selected to maintain their progeny by the presence of "natural" antigens and without a requirement for T-cell help. In contrast, B cells generated from adult bone marrow are not selected in this manner, even though they are capable of rearranging the same genes. This model of B-cell development is diagrammatically depicted in Fig. 16. The molecular basis of such physiological distinctions between fetal and adult B cells remains to be elucidated. Nevertheless, our results (and

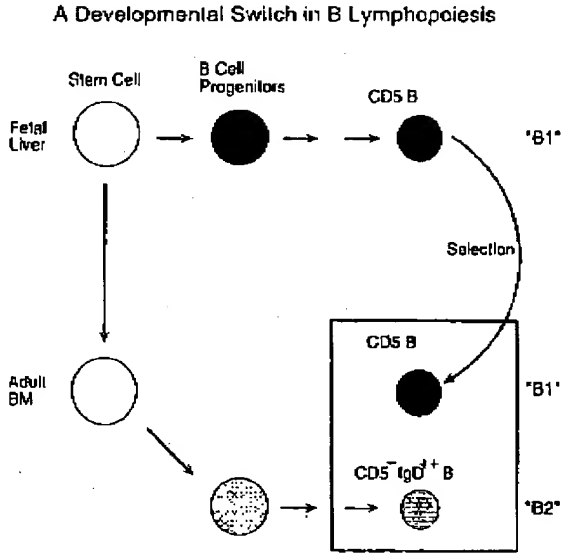

Figure 16. Proposed model of B-cell differentiation at distinct developmental timings yielding CD5$^+$ B cells (referred to as "B1") fetally and conventional B cells (CD5$^-$IgD^{hi} B, referred to as "B2") in the adult.

others") lead us to consider that fetal B cells may serve as an inherited immune system to natural (self-) antigens. Within this population, quantitative increases of B cells with useful specificities could provide the mechanism for a type of "memory" maintained through life.

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Sex Hormones as Negative Regulators of Lymphopoiesis

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SUMMARY

B lymphocytes, together with cells of seven other lineages, are made in large numbers from precursors in the bone marrow. Using cell culture models and recombinant proteins, progress has been rapid in identifying cytokines which could potentially regulate the proliferation, differentiation and migration of B-cell precursors. However, we still know little about molecular mechanisms which are important for maintaining steady-state conditions *in vivo*. B lymphopoiesis is severely diminished during pregnancy in normal mice and this provided a clue that sex hormones might be important negative regulators. Administration of estrogens alone, or in combination with progesterone, preferentially suppressed IL-7 responding cells and their progeny in bone marrow. There is precedent for these observations in the thymus, which transiently involutes during pregnancy, and also atrophies following estrogen treatment. The actual mechanism(s) through which sex steroids influence lymphopoiesis remain unclear, but cell culture experiments should be informative about potential interactions between hormones, the bone marrow microenvironment, and lymphocyte precursors. These findings raise a number of other important issues. For example, we need to learn if sex steroids are produced and/or concentrated locally within the marrow, if human lymphopoiesis is sensitive to these hormones, and if production of lymphocytes can be augmented in aging and in immunodeficiency by hormone manipulation.

INTRODUCTION

Blood cell formation initiates during embryonic development and eventually becomes established within the bone marrow. Thereafter, the production of these

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